METHODS

Mouse strains. Ago2 insertional mutant mouse strains, generated previously[20], were used for mutant analysis, ES cell derivation and reporter analysis. Ago1 gene trap strain was generated through germline transmission of Ago1 gene trap ES cells from Bay Genomics (RRR031). Ago2 catalytically inactive mutant knock-in mice were generated through germline transmission of positive ES cell clones targeted with a bacterial artificial chromosome (RP23-56M12) that has been modified with a point mutation D598A in the PIWI domain of Ago2.

Beta-galactosidase staining. For whole-mount staining, embryos from different stages were dissected together with their extra-embryonic compartments in PBS. Beta-galactosidase staining was performed using Millipore's staining reagents. X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) staining was performed overnight at room temperature. For placental sections, whole placentas were first stained for beta-galactosidase, sectioned and counterstained with haematoxylin and eosin.

Ago2 mutant crosses and ES cell derivation. Ago2 mutant phenotype was re-examined combining two insertional alleles for ease of genotyping the homozygous progeny and to take advantage of the Ago2 beta-galactosidase reporter allele. Ago2 null ES cells were derived as described previously[50]. Null cells were genotyped using primers specific to both insertional alleles. $Ago2^{mc}$ allele: forward (GACGGTGAAGAAGCACAGGAA), reverse (GGTCCGATGGGAA AGTGTAGC). $Ago2^{gt}$ allele: forward (ATGGGATCGGCCATTGAA), reverse (GAACTCGTCAAGAAGGCG).

RT–PCR, western blot and immunoprecipitation. Ago2 RT–PCR primers were designed downstream of both insertional alleles: Ago2F: TGTTCCAGCA ACCTGTCATC, Ago2R: GATGATCTCCTGTCGGTGCT. Actin primers were used as a normalization control. ActinF: ATGCTCCCCGGGCTGTAT, ActinR: CATAGGAGTCCTTCTGACCCATTC. Quantitative RT–PCR was performed using Invitrogen SuperScript III and Applied Biosystems SYBR green PCR reagent. miRNA levels were measured using Applied Biosystems pre or mature miRNA assays. Ago2 western blot and immunoprecipitation analysis were performed using Abnova anti-eif2c2 antibody (M01). P53 western was performed using Santa Cruz mouse monoclonal antibody (Pab240).

ES-tetraploid aggregation. Ago2 null ES cells were injected into tetraploid blastocyst as described previously[51]. Embryos were transferred to foster mothers and dissected at embryonic day 12.5. Beta-galactosidase staining was performed as described above.

Peripheral blood collection and FACS analysis. Blood was collected from decapitated fetuses (pre-mortem) using heparinized microcapillaries and the complete blood count was performed using a Hemavet. For FACS analysis, single cells were isolated from neonatal liver, spleen and bone marrow and co-stained with Anti-Ter-119 and CD71 antibodies (BD) and analysed on LSRII flow cytometer (BD) as described previously[52]. The same number of events of each sample were collected according to doublet discrimination gating and analysed as follows: the ProE cell population was defined by CD71 high/Ter-119 medium positive events. The Ter-119 high population was further subdivided into basophilic, late basophilic/polychromatic and orthochromatic/reticulocyte cell populations according to CD71 and forward scatter parameters to define the subsequent differentiating erythroblasts[53].

Small RNA cloning and bioinformatics annotation. Total RNA was extracted from embryonic day 18.5 livers using TRIzol. Two small RNA libraries with a size range of 19–30 nucleotides were generated using a modified small RNA cloning strategy[48,54]. Briefly, the small RNA fraction was ligated sequentially at the 3′OH and 5′ phosphates with synthetic linkers, reverse transcribed and amplified using Solexa sequencing primers. Around 7 million reads were generated for each small RNA library. Sequences were then trimmed from the 3′ linker, collapsed and mapped to the mouse genome with no mismatches using several annotation tracks, namely: UCSC genes, miRNAs and repeats. For this study we used the miRBase database to annotate the cloned miRNAs. Raw data and annotated sequences of the small RNA libraries are uploaded in the GEO database (accession number GSE21370).

Cell culture, plasmids, transfections and sensor assays. miR-144-451 expression vector was constructed by cloning the genomic cluster into pMSCV retroviral vector. Cre-ER MEFs and ES cells were cultured as described previously[50]. Excision of the *Dicer* and *Drosha* alleles was mediated through tamoxifen treatment (100 nM) for 5 days followed by transient transfection of miR-451 expressing plasmid using lipofectamine (Invitrogen). For *in vitro* processing assays and northern blots 293T cells were cultured in DMEM medium with 10% FBS serum and cotransfected using LT-1 Mirus reagent with Flag-tagged Drosha and DGCR8 constructs, Myc-tagged Ago2 or Ago1 with MSCV-miR-144-451 expression vector or Myc-tagged Ago2 alone. Dual luciferase assays were performed as described previously. For validation of the Ago2 null ES cells, a luciferase plasmid with no artificial site was cotransfected with a perfectly matched siRNA duplex (Dharmacon). Testing the functionality of miR-451 mimics was performed using three strategies: (1) cotransfection of let-7c-miR-451 mimics, pre-let-7c or let-7c duplex or control RNAs (Dharmacon) at a 100 nM concentration with let-7c luciferase reporter construct containing two perfect matching sites in the 3′UTR in HEK293 cells. (2) Similarly, tetracycline-inducible let-7c GFP sensor ES cells containing two perfectly matched sites cotransfected with PE-labelled siRNA and let-7c-miR-451 mimics (50 nM) followed by GFP analysis of PE-positive cell population using LSRII flow cytometer (BD). GFP sensor was induced using doxycycline (1 μg ml^{-1}). (3) For p53 knockdowns, ES cells were transfected with p53 shRNA and p53-miR-451 mimics followed by p53 induction using adriamycin (0.5 μg ml^{-1}) within the last 8 h before collection. All cells were collected 48 h post-transfection.

Drosha and Dicer *in vitro* processing assays. PCR fragment mapping to *miR-451* and *miR-144* were amplified out of the human genome with T7 promoter sequence. Pre-miR-451 and pre-miR-144 RNA transcripts were generated using the genomic PCR product and Ambion's T7 *in vitro* transcription kit. Transcripts were gel-purified and used in a Drosha *in vitro* processing assay as described previously[49,55]. For the Dicer *in vitro* processing assay, ^{32}P end-labelled synthetic pre-miR-451 and pre-let-7c was incubated with one unit of human recombinant Dicer (Genlantis) in 30 mM Tris/HCL pH 6.7, 50 mM NaCl and 3 mM $MgCl_2$ buffer at 37 °C over a 4 h time course.

RNA northern blot analysis. RNA was extracted from liver homogenates and Ago2 immunoprecipitates using TRIzol reagent. Total RNA (10–15 μg) and half of the immunoprecipitated RNA was run on a 20% acrylamide gel and transferred onto a positively charged nylon membrane (Hybond). Membranes were crosslinked, pre-hybridized in ultra-hyb solution (Ambion) and hybridized with ^{32}P-labelled DNA probes complementary to miR-451 and let-7c. Membranes were washed with 2×SSC, 0.1% SDS and 1×SSC, 0.1% SDS buffer and exposed on a phosphorimager screen overnight.

Ago2 cleavage assays and beta elimination. Ago2 Myc-tagged constructs (WT and D597A) were transfected in 293T cells. Lysates were collected after 48 h, immunoprecipitated using Myc agarose beads. The catalysis reaction was carried out on beads using 5′ ^{32}P end-labelled synthetic pre-miR-451 (Dharmacon) as described previously[20]. Beta elimination was performed by treating the purified RNA from the Ago2 beads with sodium periodate for 30 min at room temperature followed by ethanol precipitation. The RNA was resuspended in loading buffer containing TBE and run on a 20% acrylamide gel where the beta elimination reaction occurs.

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